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Aldolase of Lactic Acid Bacteria: a Case History in the Use of an Enzyme as an Evolutionary Marker

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INTRODUCTION

The first serious attempt to classify bacteria was undertaken by Ferdinand Cohn in 1872 (16). Although his system of classification is still recognized as a scholarly work (77, 78), Cohn's lasting contributions to bacterial taxonomy can be found in his penetrating philosophical discussions of this discipline (16, 17). The binomial system of nomenclature adopted by Cohn for prokaryotes was similar to that used for plants and animals; however, he was careful to point out that fundamental differences existed between the two systems of classification. Unlike the higher forms of life, bacteria exhibit developmental stages of so rudimentary a kind that they cannot serve as guidelines for arranging them in a natural or phylogenetic system. Cohn emphasized this point and the fact that his classification scheme was based exclusively on the morphological characteristics of the representative bacterial types by referring to his genera as "form" genera. The accretion of newly described species of bacteria compelled Migula (51) to modify and enlarge Cohn's classification system. He used physiological as

well as morphological traits to differentiate the ever increasing number of recognizable bacterial species; in doing so, Migula established a precedent which is still followed today. Orla-Jensen (57, 58) relied even more heavily on the use of nutritional and biochemical traits to differentiate the taxonomic groups in his classification system.

Taxonomic schemes grew apace with the expanding biochemical and nutritional literature and eventually evolved into determinative keys which carefully avoided all allusions to natural relationships. However, once it was realized that bacteria possess a limited number of common biosynthetic and energy-producing pathways (37), the notion occurred to microbiologists (38, 77) that the grouping of microorganisms in accordance with their major physiological traits may represent a gross sort of natural classification. Two major scientific discoveries, a chemical interpretation of the structure of the genome (80) and the deciphering of the genetic code (55), provided a means by which these intuitive systems of classification could be verified. The role of the chromosome as regulator of the various development processes in plants and animals had been appreciated, if not fully understood, since the work of Mendel (50), and the integration of this body of knowledge with the newly discovered chemical basis of gene function presented the first opportunity to compare simple or complex forms of life at the most fundamental level yet attained. By determining the degree of physical homology between two or more types of microbial deoxyribonucleic acid (DNA), the taxonomist could now precisely measure the extent of phylogenetic relatedness among those bacteria from which the genome or genome segments had been isolated.

Within the last decade, however, the limitations of this technique have become apparent, and thus far, only interspecific (23, 35, 52, 68) and, in one instance, intergeneric relationships (11) have been established among large families of bacteria thought to have natural affinities. Hybridizations between DNA and ribosomal ribonucleic acid (rRNA) as suggested by Mandel (45) and Stanier (71) will probably circumvent the restrictions imposed by the use of DNA duplexes, since rRNA appears to be highly conserved. The failure to demonstrate DNA homology above the species or genus level can probably be attributed to the degenerate and redundant nature of the genetic code (20, 21, 24, 34, 69), whereby the structure of the genome, as manifested by the nucleotide base sequence, can change significantly without altering the hereditary information contained therein. If the genetic message is, in fact, conserved, then it follows that the structure of the gene product is conserved to the same extent. Comparative studies of isofunctional proteins of higher organisms have revealed that in many instances structural homology still exists in evolutionarily diverse organisms (2, 4, 24, 54, 84, 85). Elaborate phylogenetic classification systems based on protein homologies have been created and these, not surprisingly, are concordant with the classification schemes based on paleontological studies.

Using much the same rationale as the biochemical evolutionists, microbiologists began applying the same techniques to problems of bacterial evolution. These began with comparative physical studies of specific enzymes in which such properties as protein charge (8, 9, 25, 33, 83), kinetic characteristics (65, 74), or regulatory mechanisms (32, 40) were compared. Although such studies yield useful information which aids in the differentiation of certain microbial genera and species, in contrast with DNA or protein homology studies, there is no way the data can be quantitated to provide a means of accurately assessing intra- or intertaxon differences. Recently, it has been suggested (42) that studies of physical properties of proteins be combined with structural homology studies; such studies will provide a firm foundation upon which natural relationships of prokaryotes can be based. One of the major benefits to be derived from a natural system of classification is that it will enable taxonomists to evaluate the accuracy of the more utilitarian determinative keys. Stanier (71) summarized this point in the following fashion: "When sufficient evidence about a particular phenotypic group has been accumulated at both the genetic and epigenetic levels, I believe that we shall be in a position to decide without much ambiguity in how far the phenotype resemblances among its members are the consequence of evolutionary filiation, and in how far they are expressions of convergence."

In this report, we discuss some of the ways in which contemporary biochemical and immunological techniques are being used to create a natural system of classification for bacteria. The pitfalls and limitations of these procedures are also discussed.

MATERIALS AND METHODS

Organisms

The species of bacteria used in this study and their sources are listed in Table 1. Whenever possible a typical representative of each species was selected for this work.

TABLE 1. *Strain designations and sources of microorganisms*

Organism	Source
Genus *Streptococcus*	
S. agalactiae	ATCC 13813
S. asalignus	ATCC 8059
S. bovis	ATCC 9809, 15351
S. cremoris	ATCC 19257
S. diacetilactis	ATCC 11007
S. durans	ATCC 19432
S. dysgalactiae	ATCC 9926
S. equi	ATCC 6580
S. equinus	ATCC 9812
S. equisimilis	ATCC 9542
S. faecalis	NIH, MR: R. Deibel, N83; E. Sharpe, C1; van Niel, H.3.1
S. faecium	E. Sharpe, K6A, CHI, N55
S. lactis	ATCC 19435
S. mitis	ATCC 15909, 15910, 15912
S. mutans	NIH, K1R, SL1, 01H1
S. pneumoniae	ATCC 6308
S. pyogenes	ATCC 14289
S. salivarius	ATCC 13419, 9222; NIH 112
S. sanguis	ATCC 10556
Streptococcus sp. (group H)	ATCC 8144
Streptococcus sp. (group L)	ATCC 9932
S. thermophilus	ATCC 19258
Genus *Pediococcus*	
P. acidilactici	ATCC 25740, 25741, 25742, 25743
P. cerevisiae	ATCC 8042
P. homari	ATCC 10400
P. parvulus	ATCC 19371
P. pentosaceus	ATCC 25744
Pediococcus sp.	E. Garvie, 559, 990
Genus *Lactobacillus*	
L. acidophilus	ATCC 4356, 19992
L. brevis	ATCC 14869
L. bulgaricus	ATCC 11842
L. casei	Rogosa, OC91, OC45, C1-11, C1-15 Gasser, 64H Kandler, M40 van Niel, F.3.2, F.3.3, F.3.4
L. coryniformis	Kandler, M30, M34
L. curvatus	Kandler, M1
L. cellobiosus	ATCC 11739
L. delbrueckii	ATCC 9649
L. fermentum	ATCC 14931
L. helveticus	ATCC 15009
L. jensenii	ATCC 25258
L. jurgurti	ATCC 5214
L. lactis	ATCC 12315
L. mali	ATCC 27053
L. plantarum	ATCC 14917
L. leichmannii	ATCC 4797
L. salivarius	ATCC 11741
L. xylosus	ATCC 15577
L. zeae	ATCC 15820
Other organisms	
Arthrobacter globiformis	ATCC 8010
Bifidobacterium asteroides	ATCC 25910
B. bifidum	ATCC 15696
B. indicum	ATCC 25912
Corynebacterium xerosis	ATCC 7094
Microbacterium thermosphactum	ATCC 11509
Sporolactobacillus inulinus	ATCC 15538
Staphylococcus aureus	ATCC 12600

Cultivation and Maintenance of the Organisms

With the exception of members of the genus *Bifidobacterium*, stock cultures of all lactic acid bacteria (lactobacilli, streptococci, and pediococci) were carried in the reinforced litmus milk medium of Rogosa (private communication), which contains 10% (wt/vol) skim milk powder, 0.5% (wt/vol) yeast extract, 0.5% glucose, 0.075% (wt/vol) litmus (Difco), and 7.1% (wt/vol) $CaCO_3$. Cultures were incubated for 18 h at 30 or 37 C and were subsequently stored at 4 C. The bifidobacteria were cultivated in thiol medium (Difco) at 37 C.

Material for small-scale enzymological experiments was obtained by growing the various organisms in 500-ml screw-capped bottles or in 1-liter Erlenmeyer flasks containing 400 ml of the appropriate medium at 30 or 37 C. The lactobacilli and homofermentative pediococci were grown in MRS medium (Difco); most of the streptococci were grown in a complex medium which has been described elsewhere (41). *Streptococcus mutans* was grown in the complex medium supplemented with 0.05% (wt/vol) Tween 80, whereas *Streptococcus* (*Diplococcus*) *pneumoniae* was cultivated in heart infusion broth (Difco) supplemented with 0.5% (vol/vol) horse serum and 1% (wt/vol) glucose. The complex streptococcal medium was also used for the cultivation of 400-liter batches of *Streptococcus faecalis* strain MR. Centrifugation of the cultures yielded cell pastes which provided the starting material for large-scale purification of the fructose diphosphate (FDP) aldolase.

All of the other microorganisms were grown and maintained according to procedures set forth in the American Type Culture Collection catalogue (10th edition).

Purification of the FDP Aldolase

Cell-free extracts of *S. faecalis* MR were prepared by the sonic disruption of 25 g (wet weight) of cells suspended in 25 ml of 0.05 M bis-Tris [2.2-bis(hydroxymethyl)-2,2',2'-

nitrilotriethanol] buffer (Aldrich Chemicals), pH 6.5, containing 10 mM β-mercaptoethanol (BTME). The crude extract was centrifuged for 30 min at 30,000 × *g*, and the clarified supernatant was decanted and centrifuged for 60 min at 100,000 × *g*. An acid precipitate was obtained by dialyzing the crude extract supernatant against 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride-acetate buffer, pH 4.5, for 12 h at 4 C. The resultant precipitate was removed by centrifugation and discarded. After the pH of the supernatant fluid was adjusted to 6.5 by the addition of 2 N NaOH, 0.05 volumes of M $MnCl_2$ were added to the extract and the resultant precipitate was removed by centrifugation and discarded. By maintaining a temperature of 4 C, sufficient $(NH_4)_2SO_4$ was slowly dissolved in the supernatant fluid to give a concentration of 60% of saturation. After the removal of the precipitated protein, the $(NH_4)SO_4$ concentration of the supernatant fluid was increased to 80% of saturation. The protein precipitate was removed by centrifugation, dissolved in 10 to 15 ml of BTME, and dialyzed for 12 h against 1 liter of the same buffer.

The dialyzed protein solution was applied to a DE23 (Reeve-Angel) column (2.5 by 40 cm) which had been equilibrated with BTME, and the enzyme was eluted with BTME and a linear 0- to 0.6-M KCl gradient. FDP aldolase was eluted from the DE23 column by 0.38 M KCl. After the fractions containing the highest levels of enzyme activity were pooled, the volume of the resulting solution was reduced to 5 ml by concentration in a 10-M Amicon filtration apparatus, and the concentrate was applied to a Sephadex G-200 upward-flow column (2.5 by 90 cm). Enzyme activity was eluted with BTME buffer containing 0.1 M KCl. The peak activity fractions were pooled and reduced in volume for further treatment.

The Buchler preparative polyacrylamide gel (PAG) electrophoresis apparatus was used to obtain samples of FDP aldolase which were electrophoretically pure. Between 3 and 5 ml of the concentrated Sephadex column eluate (30 mg of protein) were layered on a 10% PAG column (9 cm long) prepared with the neutral buffer system described by Chrambach and Rodbard (13). A current of 22 mA was applied to the column, and 5-ml fractions were collected from the cathodal end of the column. This procedure resulted in a loss of from 40 to 50% of the enzyme activity, whereas electrophoresis of eluates in conventional anionic buffer (22) caused a 90% inactivation.

Storage of Purified Aldolase and Cell-Free Extracts

Both in the pure state and in cell-free extracts, the *S. faecalis* aldolase was extremely sensitive to freezing and thawing. Three cycles of this treatment resulted in a 90% loss of the initial activity. The addition of 20% (vol/vol) glycerol stabilized the enzyme in solution and permitted repeated freezing and thawing with no detectable loss of activity. Thereafter, glycerol was routinely added to all aldolase preparations prior to storage at −40 C.

Disc Gel Electrophoresis

The electrophoretic mobility rates of the various lactic acid bacteria aldolases were measured with a Buchler disc gel polyanalyst by using the anionic buffering system of Davis (22) or the neutral buffering system cited earlier. From 25 to 60 μg of crude extract protein or 5 to 20 μg of pure streptococcal aldolase were layered on PAG columns, and a current of 2.5 mA was applied to each column. Protein was detected by staining with Coomassie blue (43). The position of FDP aldolase was located by bathing the PAG column in 5 ml of a solution containing: 0.05 M Tris-hydrochloride buffer (pH 7.3), 0.01 M potassium acetate; 0.3 mM β-mercaptoethanol; 0.001 M nicotinamide adenine dinucleotide (NAD), 0.02 M FDP, 0.035 mg of triosephosphate isomerase (Sigma), 0.5 mg of 3-phosphoglyceraldehyde dehydrogenase (Sigma), 0.01 M sodium arsenate, 0.004 mg of phenazine methosulfate, and 1 mg of nitroblue tetrazolium.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out by the procedure of Weber and Osborne (81).

Enzyme Assays

FDP aldolase activity was determined spectrophometrically by using the method of Groves et al. (30). A unit of activity is defined as the amount of enzyme required to oxidize 1 μmol of reduced NAD per min. The order of addition of ingredients in the assay mixture was generally not critical. However, the usual practice of starting the reaction with the addition of substrate resulted in the complete inhibition of the aldolase activity of *Lactobacillus plantarum* cell-free extracts. Aldolase activity was only detectable when the cell-free extract was used to initiate the reaction.

Molecular Weight Determination

The molecular weights of the various aldolases were estimated by the molecular sieve

technique of Andrews (3) by using an upward-flow Sephadex G-200 column which had been standardized with ribonuclease, chymotrypsinogen A, ovalbumin, bovine serum albumin, muscle aldolase, and human 7S gamma-globulin. The estimation of the molecular weight was independent of the protein concentration within the range used in these experiments.

Preparation of the Streptococcal Antialdolase Serum

Four 6-month-old male New Zealand white rabbits each received a series of four intradermal injections consisting of 0.2 ml of electrophoretically pure *S. faecalis* MR FDP aldolase (420 μg), 0.2 ml of complete Freund's adjuvant (Difco), and 0.01 ml of 1% methylated bovine serum albumin (MBSA) over a 4-week period. These were followed by two intravenous injections consisting of 0.25 ml of aldolase and 0.01 ml of 1% MBSA given at 10-day intervals. The levels of aldolase-specific antibodies produced by the rabbits during the course of immunization were monitored by semiquantitative precipitin tests. The rabbits were bled from the central ear artery 1 week after the final intravenous injection, and the blood was stored at 4 C overnight. The clotted erythrocytes and fibrin were removed by centrifugation, and the antistreptococcal aldolase serum (anti-SA) was divided into 5-ml samples and stored at −40 C until used.

Immunoelectrophoresis

One of the methods used to establish the purity of the *S. faecalis* FDP aldolase was immunoelectrophoresis. The technique has been described previously (43), and the only modification in the present procedure involved the use of 0.04 M Tris-hydrochloride, pH 8.8, instead of 0.04 M barbitol buffer, pH 8.2. The upper well of the agar plate received 12 μg of pure aldolase, while the lower well was charged with 40 μg of crude extract protein. After passing a current of 5 mA through the plate for 75 min, the center trough received 40 μliters of a 1:5 dilution of anti-SA. The plate was incubated for 18 h at 4 C in a high-humidity chamber; the results are shown in Fig. 1. With either source of antigen, only a single precipitin line was formed, indicating that the purified aldolase was free of contaminating protein.

Immunodiffusion

Immunological homology between the various lactic acid bacteria aldolases and the *S. faecalis* MR aldolase was first detected by using the Stollar and Levine (75) modification of the Ouchterlony immunodiffusion method. Minor modifications of the procedure are published elsewhere (44). The sample wells were charged with solutions containing from 0.1 to 0.8 units of aldolase activity, whereas the center well received sufficient anti-SA to produce sharp lines of precipitation. The convention of Gasser and Gasser (26), the essential points of which are described below, was used to collate and summarize the immunodiffusion data.

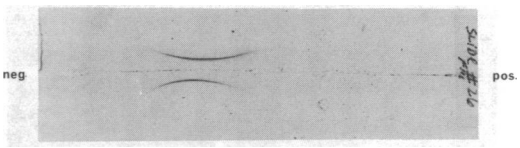
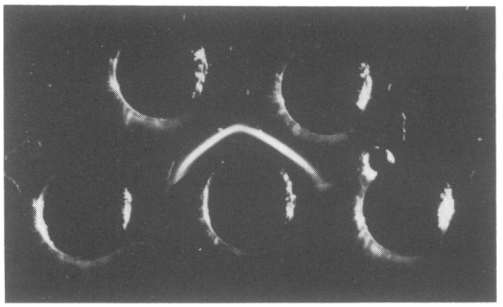


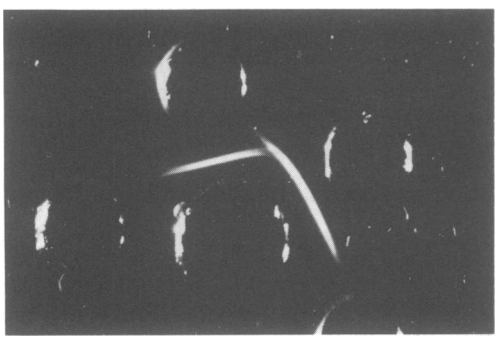
FIG. 1. *Protein stain of antigen-antibody complex on immunoelectrophoresis plate. The upper well contains electrophoretically pure S. faecalis MR aldolase; the lower well contains crude S. faecalis MR extract.*

Interpretation of Immunodiffusion Results

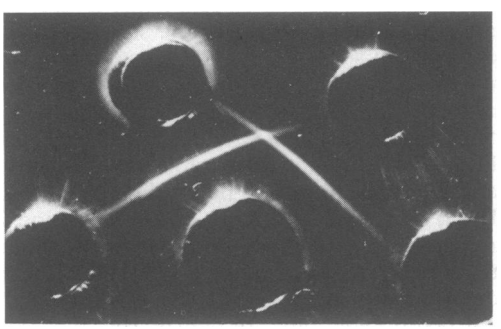
The theoretical aspects of immunodiffusion as they apply to this study have been discussed recently by Gasser and Gasser (26) and require no detailed elaboration here. However, a brief description of the three basic precipitation patterns and their significance will greatly simplify the presentation of the data. If the interactions of the reference (homologous) protein, an unknown protein, and the antiserum prepared against the homologous protein produce fused or confluent lines of precipitation (Fig. 2A), the unknown protein possesses the same antigenic determinants as the homologous protein and the two form a group of *identical specificity*. It is also possible for two or more heterologous proteins to interact and produce fused precipitin lines. For example, if heterologous proteins 1 and 2 bearing antigenic determinants, A, B, C, D, U, V, and W and A, B, C, D, X, Y, and Z, respectively, are made to react with an antiserum prepared against a protein with the determinants A, B, C, D, E, F, and G, the antibodies will only recognize the first four antigenic sites of the heterologous proteins and produce confluent precipitates. Such proteins are assigned to groups of *apparent identical specificity*. A precipitate with a single spur is produced when one of the two antigens being compared has more determinants in common with the homologous antigen than its neighbor (Fig. 2B). This pattern is known as a reaction of *partial identity* and usually occurs when two or



A.



B.



C.

FIG. 2. *Three basic types of immune precipitates. A, Confluent lines or reaction of identity. B, Single spur or reaction of partial identity. C, Double spurs or reaction of nonidentity.*

more proteins have evolved in a sequential or unidirectional fashion. The double-spurred precipitate or reaction of *nonidentity* results from the interaction of two heterologous proteins which share a number of antigenic determinants with the homologous protein but very few or none with each other (Fig. 2C). This pattern indicates that the two heterologous proteins have evolved in a random or divergent fashion with respect to the reference protein.

Microcomplement Fixation

The microcomplement fixation assay (mCf) of Wasserman and Levine (79) provides an accurate means of measuring the immunological differences between two partially homologous proteins; however, the information content of the results is quite different from that obtained by immunodiffusion. In contrast to immunodiffusion, mCf cannot differentiate between sequential and random changes in the antigenic composition of two or more heterologous proteins because this technique measures the interaction of the antigen and antibody indirectly. The amount of complement fixed is simply a function of the number of antigenic determinants recognized by the antiserum. For example, if an antiserum prepared against a reference protein bearing the determinants A, B, C, D, E, and F is used to compare two heterologous proteins possessing the determinants A, B, C and D, E, F, respectively, the extent of immunological reactivity as measured by mCf would be very similar or identical. However, the divergent nature of these two proteins would not be detected by this test. For this reason, mCf is used in conjunction with immunodiffusion experiments to corroborate and quantitate the gross immunological variations detected by the latter.

Guinea pig complement (GPC) was standardized by the procedure of Hook and Muschel (29), and 2.5 50% complement fixation units were routinely used in the experiments. The mCf system was standardized with the pure aldolase as antigen and anti-SA; by using the former in a range of 0.1 to 0.8 μg and the latter at a dilution of 1:75,000, 80% of the added GPC was fixed at equivalence (0.3 μg of antigen). Thereafter, the source of antigen was a cell-free extract which had been first centrifuged for 30 min at 30,000 $\times$ *g* followed by centrifugation for 90 min at 200,000 $\times$ *g* to eliminate anti-complementary activity (44). Cell-free extracts containing the homologous antigen were diluted to give a range of 0.8 to 8 μg of protein; extracts of pediococci were diluted to give a range of 2 to 20 μg of protein; and extracts of lactobacilli were used in the range of 4 to 40 μg of protein. Extracts of each organism surveyed were tested at least twice at three antiserum concentrations.

The results of the mCf experiments were first analyzed to learn whether the percent of complement fixed was a linear function when compared with the log of the antiserum dilution. If the value of the slope of the line obtained with the heterologous (HET) antigen is the same as that calculated for the homologous (HOM) antigen, a direct comparison of the data can be made (67); an example of several such determinations is depicted in Fig. 3. The indices of dissimilarity (ID) were calculated for the heterologous aldolases by using the following mathematical expression derived by Wilson et al. (85).

Index of dissimilarity

$$= \frac{\text{AB dilution HOM} \times \%\text{ complement fixed HOM}}{\text{AB dilution HET} \times \%\text{ complement fixed HET}}$$

Since the ID value is an expression of the degree of antigenic homology between the homologous and heterologous aldolase, the results represent a comparative measure of structural homology.

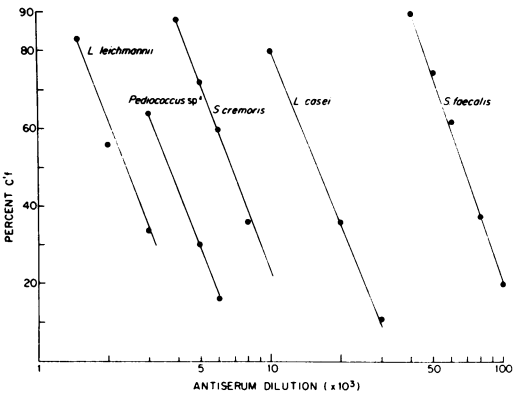


FIG. 3. *Relation of complement fixation to the log of the antibody concentration.*

RESULTS

Physical and Biochemical Properties of the FDP Aldolases of Lactic Acid Bacteria

Characterization of the pure S. faecalis aldolase. The purified aldolase exhibited maximum activity at a pH of 7.5. At this pH, the apparent K_m for the substrate, FDP, is 0.8 mM. Addition of 25 mM NH_4Cl produces a twofold increase in the reaction rate in the presence of 20 mM KCl. A kinetic analysis of the data reveals that the ammonium salt only increases the maximum velocity of the reaction, the K_m, for FDP is not altered. As is the case with other class II aldolases (65), ethylenediaminetetraacetic acid (EDTA) inhibits catalytic activity; however, the inhibition is unconventional in that the enzyme and EDTA do not interact immediately. To produce a 50% inhibition of aldolase activity, the enzyme had to be preincubated with 0.1 mM EDTA for 15 min prior to the addition of substrate.

A number of nucleotide phosphates and intermediate products of glucose catabolism were tested as potential regulators of the FDP aldolase. The following nucleotide phosphates —adenosine 5'-triphosphate (ATP), deoxy ATP, adenosine diphosphate, adenosine 5'-monophosphate, guanosine 5'-triphosphate, cytidine 5'-triphosphate, and inosine 5'-triphosphate—had no demonstrable effect on aldolase activity in the range of 2.5×10^{-5} M to 1×10^{-3} M. Similarly, at concentrations of 5×10^{-4} M to 1.5×10^{-2} M glucose-6-phosphate, fructose-6-phosphate, 6-phosphogluconate, ribose-5-phosphate, xylose-5-phosphate, pyruvate, and phosphoenol pyruvate neither enhanced nor inhibited the enzyme activity. Acetyl coenzyme A and acetyl phosphate were also tested at levels ranging from 5×10^{-5} M to 5×10^{-3} M; neither had any effect on enzyme activity.

Compared with other aldolases (65), the streptococcal enzyme with a molecular weight of 56,000 as estimated by Sephadex G-200 chromatography is relatively small (Fig. 4). This value is concordant with that obtained by using crude cell-free extracts. While the enzyme migrates as a single protein band in SDS polyacrylamide gels (Fig. 5), the rate of migration indicates that the aldolase has dissociated into two subunits which have a molecular weight of 28,000 each (Fig. 6).

Size determinations of other lactic acid bacteria aldolases. A comparative survey of other lactic acid bacteria revealed the existence of five molecular weight classes of FDP aldolase (Table 2). These vary in molecular weight from 56,000 to 176,000, and a minimum of two weight groups has been found for each genus in the family. For example, the majority of species tested in the genus *Streptococcus* possess an aldolase which has a molecular weight of approximately 56,000; however, the aldolase of *Streptococcus salivarius* is greater by a factor of 2.5 (molecular weight = 135,000). With three molecular weight groups of aldolase (56,000, 76,000, and 118,000) found among its members, the genus *Lactobacillus* is distinguished by the diversity of enzyme size. The pediococcal aldolases, on the other hand, exhibit the greatest size difference; the aldolase of *Pediococcus parvulus* has an estimated molecular weight of 58,000,

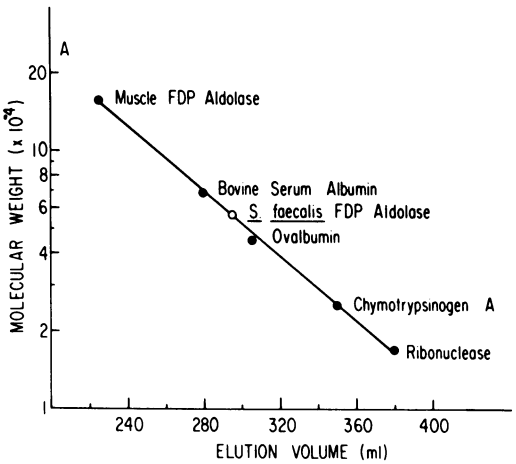


FIG. 4. *Estimation of the molecular weight of the purified S. faecalis MR aldolase by Sephadex G-200 filtration.*

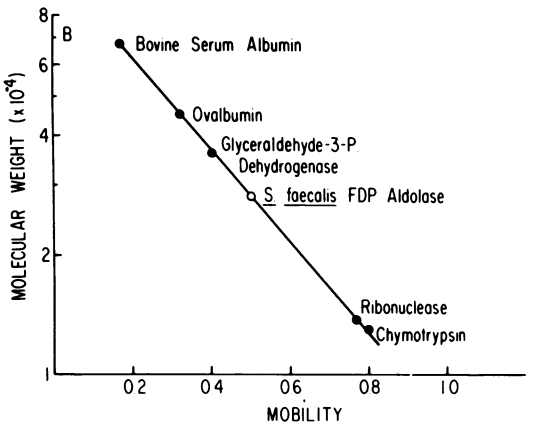


FIG. 6. *Determination of the molecular weight of the S. faecalis MR aldolase subunits by SDS-polyacrylamide gel electrophoresis.*

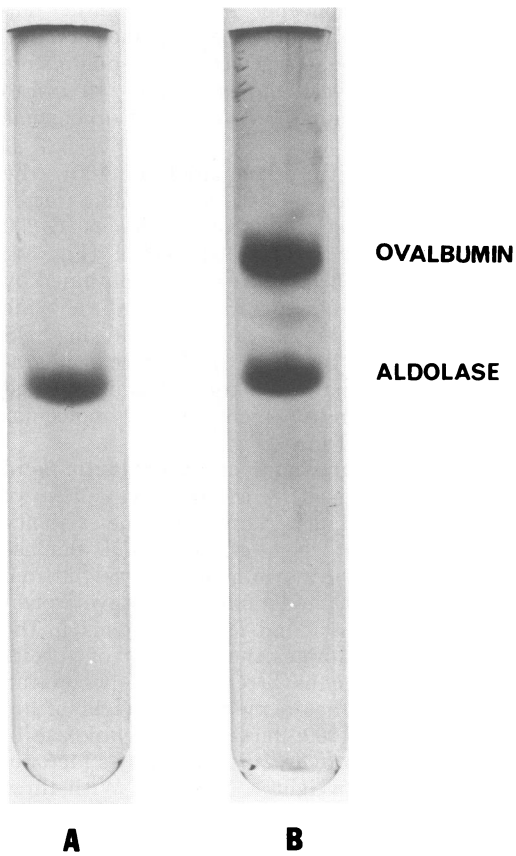


FIG. 5. *Protein stain of SDS-polyacrylamide gels showing the position of the S. faecalis MR aldolase subunits. A, Aldolase alone. B, Aldolase plus ovalbumin standard.*

whereas the enzyme from other species has an approximate molecular weight of 176,000.

Aldolase activity could not be detected in cell-free extracts of *Pediococcus homari*, *Bifidobacterium bifidum*, *Bifidobacterium asteroides*, *Bifidobacterium indicum*, and the heterofermentative lactobacilli (*Lactobacillus fermentum*, *Lactobacillus cellobiosus*, and *Lactobacillus brevis*).

Representatives of each aldolase size group were subjected to chemical treatments, which included incubation in 0.1 M acetate buffer adjusted to a pH of 4.5, heating at 56 C for 10 min, and absorption and elution from diethylaminoethyl-cellulose. None of these treatments altered the molecular weight of the respective aldolases. Furthermore, the mixing of two cell-free extracts containing a small and large aldolase, respectively, had no effect on either enzyme; Sephadex G-200 chromatography easily separated both activities.

FDP affinity constants of the five classes of aldolase. By using cell-free extracts of *S. faecalis* MR (aldolase molecular weight = 56,000), *Lactobacillus xylosus* ATCC 15577 (aldolase molecular weight = 70,000), *Lactobacillus casei* 64H (aldolase molecular weight = 118,000), *S. salivarius* 112 (aldolase molecular weight = 130,000), and *Pediococcus acidilactici* (aldolase molecular weight = 176,000), K_m values for FDP of 0.8 mM, 0.45 mM, 0.45 mM, 0.8 mM and 0.38 mM were observed with the respective enzymes. These preliminary data suggest that the affinity of the enzyme for its substrate is not a function of enzyme size. However, with the exception of the *S. faecalis* aldolase experiments, confirmation of these results must await further studies with the pure aldolases.

TABLE 2. *Molecular weights[a] of the FDP aldolases found among lactic acid bacteria*

Streptococcus	Mol wt	*Lactobacillus*	Mol wt	*Pediococcus*	Mol wt
S. faecalis (MR)	56,000	I. *L. casei* (64H)	118,000	I. *P. cerevisiae* (8042)	176,000
S. faecium (K6A)	57,000	*L. casei* (OC91)	116,000	*Pediococcus* sp. (559)	176,000
S. bovis (9809)	59,000	*L. zeae* (15820)	118,000	*Pediococcus* sp. (990)	176,000
S. equinus (9812)	58,000	*L. delbrueckii* (9649)	118,000	*P. pentosaceus* (25744)	178,000
S. dysgalactiae (9926)	58,000	*L. leichmannii* (4797)	116,000	*P. acidilactici* (25740)	176,000
S. sanguis (10556)	57,000	*L. acidophilus* (4356)	122,000		
S. mutans (6715)	64,000	*L. jensenii* (25258)	122,000	II. *P. parvulus* (19371)	58,000
S. pneumoniae (6308)	56,000	*L. helveticus* (15009)	122,000		
Streptococcus sp. (group H) 8144	56,000			Others	
				Microbacterium thermosphactum (11509)	56,000
S. salivarius (13419)	138,000	II. *L. plantarum* (14917)	78,000		
S. salivarius (112)	130,000	*L. salivarius* (11741)	80,000	*Sporolactobacillus inulinis* (15538)	56,000
S. salivarius (9222)	135,000	*L. xylosus* (15577)	70,000		
		III. *L. curvatus* (M1)	56,000		

[a] Determined by Sephadex G-200 chromatography.

Comparison of electrophoretic mobility rates of aldolases. Cell-free extracts of various lactic acid bacteria were applied to anionic PAG columns and after electrophoresis, the positions of the FDP aldolases were located by activity stains. The electrophoretic mobility constants (R_f) were calculated from the distances traveled by the respective aldolases. In some instances, only a single dye band was observed; however, in most cases a major dye band and one or more minor bands appeared. Although the R_f values of the minor components are presented in the summary of the data (Table 3), the size and intensity of the major dye bands leave little doubt that they are, in fact, the FDP aldolases. This supposition is supported by the fact that the R_f of the major dye band produced by cell-free extracts of *S. faecalis* is identical with the R_f value of the pure aldolase. The nature of the minor components is still unknown, and although they may be nonspecific dye-reducing proteins, it is also possible that they are isozymes or dissociated aldolase subunits.

The data presented in Table 3 indicate that the aldolases can be assigned to one of four electrophoretic mobility groups; these divisions can be seen more clearly in the diagrammatic presentation of the results (Fig. 7). Apart from the streptococci, the intrageneric groupings based on the aldolase R_f values coincide with the molecular weight divisions; that is, the smaller aldolases of the lactobacilli and pediococci migrate more rapidly than the large enzymes. Surprisingly, the larger *S. salivarius* enzyme migrates at roughly the same rate as the smaller streptococcal aldolases.

Temperature sensitivity of the aldolases. It is apparent from the preceding results that, within each molecular weight group, many of the enzymes have readily detectable charge differences. Since charge differences are usually indicative of amino acid substitutions within the respective proteins, an attempt was made to correlate the mobility coefficients and molecular weights with a third property of the enzymes, thermostability. The simple procedure is appended to Table 4, where the experimental results are summarized. No clear relationship between response to heat and protein charge or molecular weight was found. The wide variations in heat sensitivity which were observed within each molecular weight class of aldolase precludes the formulation of any general hypothesis relating enzyme size to heat response. Likewise, the protein charge differences cannot be used to predict whether a particular enzyme is heat sensitive or resistant; the amino acid mutations which confer heat stability upon an enzyme apparently do not necessarily alter the protein charge.

Demonstration of Immunological Relatedness Among FDP Aldolases of the Lactic Acid Bacteria by Immunodiffusion

Separation of the streptococcal aldolases into five antigenically distinct groups. The extent of relative immunological homology between the various FDP aldolases was approximated by an extensive comparative study using cell-free extracts of strains representing 21 species of *Streptococcus* as sources of antigen.

TABLE 3. *Electrophoretic mobility rates of the FDP aldolases found among members of the lactic acid bacteria*

Species tested	Strain no.	R_f major band(s)	R_f minor band(s)
Streptococcus sp. (group H)	ATCC 8144	0.63	0.69, 0.78
S. sanguis	ATCC 10556	0.64	0.67
S. pyogenes	ATCC 14289	0.65	0.78
S. bovis	ATCC 15351	0.65	0.81
S. diacetilactis	ATCC 11007	0.68	0.75
S. bovis	ATCC 9809	0.69	0.75
S. lactis	ATCC 19435	0.69	0.77
S. mitis	ATCC 15909	0.69	0.74
S. dysgalactiae	ATCC 9826	0.72	0.81
S. equinus	ATCC 9912	0.73	0.81
S. salivarius	ATCC 13419	0.73	0.86
S. cremoris	ATCC 19257	0.73	0.80
S. faecalis	MR	0.73	0.81, 0.85
S. faecalis	C1	0.73	
S. mutans	K1R	0.74	
S. mutans	01H1	0.74	0.85
S. pneumoniae	ATCC 6308	0.75	0.43
S. salivarius	112	0.75	0.85
S. durans	ATCC 19432	0.76	
S. mitis	ATCC 15910	0.77	0.82
S. agalactiae	ATCC 13813	0.78, 0.75	0.53
S. thermophilus	ATCC 19258	0.79	0.86
Lactobacillus jensenii	ATCC 25258	0.32	
L. acidophilus	ATCC 19992	0.38	
L. jugurti	ATCC 521	0.40, 0.31	
L. casei var. *casei*	64H	0.40	0.48
L. casei var. *casei*	F.3.2	0.44	0.48
L. casei var. *rhamnosus*	OC-91	0.44	0.54
L. lactis	ATCC 12315	0.44	0.32, 0.18
L. delbrueckii	ATCC 9649	0.47	0.29, 0.15
L. bulgaricus	ATCC 11842	0.47	0.52
L. helveticus	ATCC 15009	0.47, 0.42	
L. leichmannii	ATCC 4797	0.50	0.26
L. plantarum	ATCC 14917	0.68, 0.42	0.46
L. xylosus	ATCC 15577	0.69	0.74, 0.80, 0.86
L. salivarius	ATCC 11741	0.71	0.75, 0.80, 0.86
Sporolactobacillus inulinus	ATCC 15538	0.57	
Pediococcus pentosaceus	ATCC 25744	0.45	
P. cerevisiae	ATCC 8042	0.50	
Pediococcus sp.	559	0.53	
Pediococcus sp.	990	0.55	
P. parvulus	ATCC 19371	0.62	
Microbacterium thermosphactum	ATCC 11509	0.67	

Since the antistreptococcal aldolase serum (anti-SA) had been prepared against the *S. faecalis* MR aldolase, this enzyme automatically became the point of reference for all comparisons. Only two of the three possible precipitation patterns were observed in this set of experiments; examples of confluent (fused) and single-spurred precipitin lines are shown in Fig. 8a and b and 9a, b, and c. The absence of double-spurred precipitates suggests that the streptococcal aldolases have not yet undergone any major antigenic divergence. On the basis of the paired cross-reactions, the various aldolases were assigned to one of five antigenic groups (Table 5); these are arranged in a descending order of antigenic homology. Group I is a collection of enzymes of identical antigenic specificity, whereas groups II, III, IV, and V contain aldolases of apparent identical specificity. Group IV is unique in that it contains aldolases of different molecular weights (56,000 and 135,000) which cannot be differentiated serologically. The results also show that the aldolase of *S. pneumoniae* can readily be classified with those of the other streptococci.

Antigenic grouping of the aldolases found

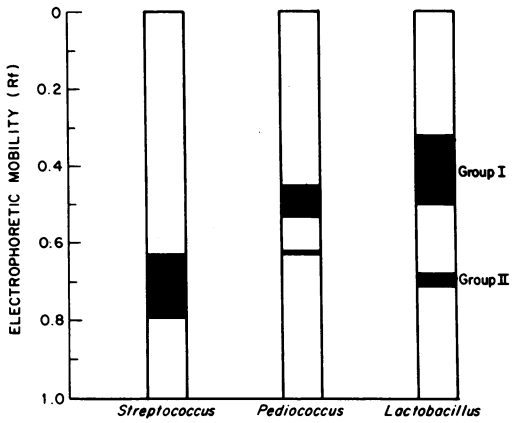


FIG. 7. *Summary of the electrophoretic mobility rates (R_f) of lactic acid bacteria aldolases.*

among the homofermentative lactobacilli. The anti-SA reacted with crude extracts of all thermo- and streptobacteria tested, indicating that some degree of antigenic homology exists between the aldolases of the lactobacilli and *S. faecalis*. Although the majority of cross-reactions produced either confluent or single-spurred precipitates (Fig. 10a, b, c), double-spurred patterns were observed in two instances. The immunodiffusion patterns segregated the homofermentative lactobacilli into nine antigenically distinct groups. The immunological hierarchy of eight of the groups is shown in Fig. 11. The species *Lactobacillus acidophilus* was divided into two groups in the order *L. acidophilus* 19992 > *L. acidophilus* 4356. These correspond to Gasser's *L. acidophilus* groups Ib and III, respectively (26). Included in this arrangement are three groups of apparent identical specificity. The aldolases of *Lactobacillus casei* var. *casei* and *L. casei* var. *rhamnosus* comprise the first group (Fig. 10). Confluent precipitin lines were also produced between extracts of *Lactobacillus leichmannii*, *Lactobacillus lactis*, *Lactobacillus bulgaricus*, and *Lactobacillus delbrueckii* (Fig. 10); *Lactobacillus jugurti* and *Lactobacillus helveticus* (not shown) exhibited a similar pattern of identity.

In contrast to the sequential precipitin reactions observed with the streptococcal aldolases, several instances of antigenic divergence were detected with aldolases of the lactobacilli. Double spurs were produced when comparing extracts of *L. acidophilus* 4356 and *L. plantarum* (Fig. 12c); the same type of reaction occurred between extracts of *L. xylosus* and *Lactobacillus salivarius* (not shown). The result of the comparison was completely unexpected since both enzymes belong to the same molecular weight class.

The constitutive nature of the FDP aldolase precluded any direct demonstration of antiserum specificity using cell-free extracts of homofermentative lactic acid bacteria. An in-

TABLE 4. *Effect of heat on the stability of FDP aldolases from lactic acid bacteria*[a]

Organism	Remaining activity (%) after 15-min incubation at:	
	48 C	56 C
Streptococcus faecalis MR	0	0
S. agalactiae ATCC 13813	5	0
S. durans ATCC 19432	65	0
S. lactis ATCC 19435	90	0
S. thermophilus ATCC 19258	84	44
S. bovis ATCC 9808	96	52
S. mutans 01H1	86	85
S. salivarius 112	90	92
Pediococcus pentosaceus ATCC 25744	50	0
Pediococcus sp. 559	95	2
P. acidilactici ATCC 25740	96	7
Lactobacillus lactis ATCC 12315	100	0
L. xylosus ATCC 15572	81	13
L. casei 64H	100	98

[a] Procedure: 0.5 ml of crude extract containing 10 to 20 units of aldolase activity were mixed with 0.5 ml of 0.05 M BTME buffer, and a zero time sample of 0.1 ml was removed and mixed with an equal volume of the BTME buffer. The remaining solution was then incubated in a water bath which was maintained at 48 or 56 C, and 0.1-ml samples were removed every 3 min for 15 min. The samples were diluted with 0.5 ml of chilled BTME buffer and rapidly cooled to 4 C. Approximately 10 μliters of the samples was subsequently assayed for aldolase activity.

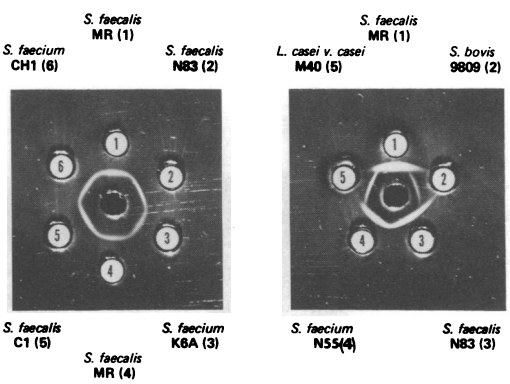


FIG. 8. *Nature of the cross-reaction between various aldolases of lactic acid bacteria.*

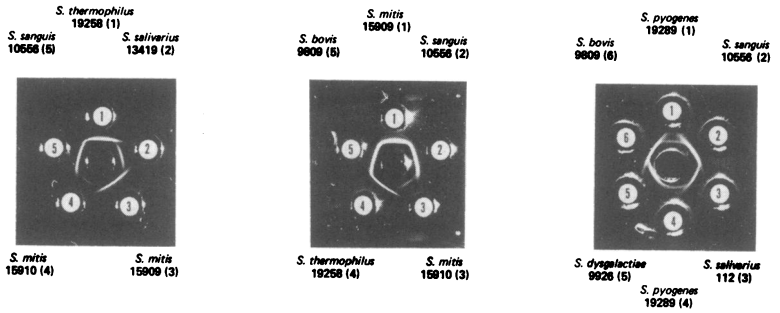


FIG. 9. *Immunological comparison of eight streptococcal aldolases. Experiments such as these were used to determine the antigenic hierarchy of the streptococci.*

TABLE 5. *Hierarchical grouping of aldolases of representatives of the genus Streptococcus based on precipitin cross-reaction with S. faecalis antialdolase*

Specificity group	Organisms
I	*Streptococcus faecalis*
	S. faecium
	S. durans
II	*S. lactis*
	S. cremoris
	S. diacetilactis
	S. thermophilus
	S. mitis (I)
III	*S. bovis*
	S. equinus
	S. pneumoniae
	S. sanguis
	S. mutans
IV	*S. salivarius*
	S. agalactiae
	S. mitis (II)
	Streptococcus sp. (group H)
V	*S. pyogenes*
	S. dysgalactiae
	Streptococcus sp. (group L)

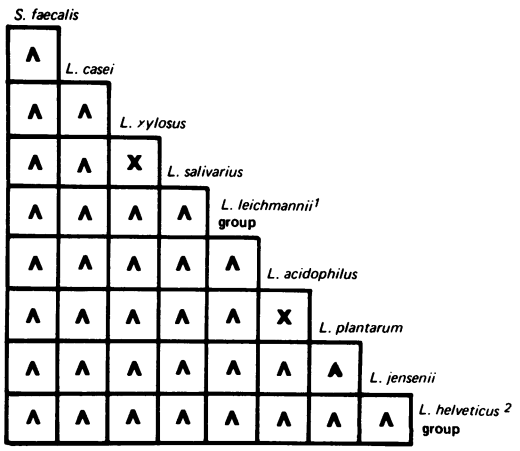


FIG. 11. *Hierarchy of immunological relatedness of Streptobacterium and Thermobacterium FDP aldolases based on precipitin cross-reactions with S. faecalis antialdolase. The arrowheads indicate the dominant antigen of the paired cross-match; the X indicates double-spur formation.*

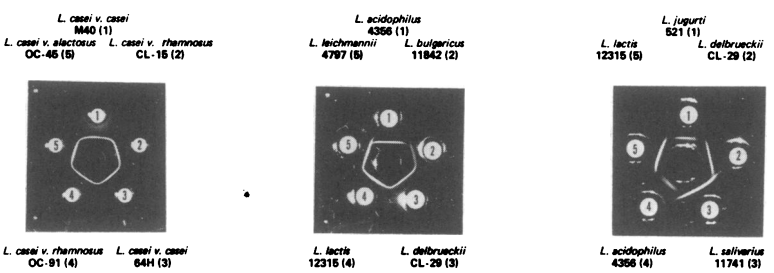


FIG. 10. *Reactions of apparent identical specificity and partial identity between aldolases of the lactobacilli.*

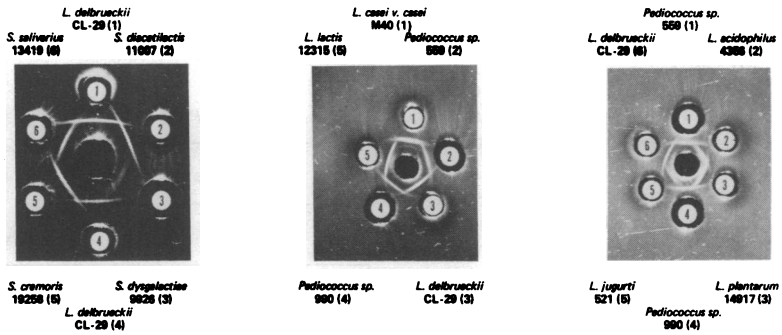


FIG. 12. *Demonstration of antigenic divergence among the three genera of lactic acid bacteria by immunodiffusion.*

direct test was conducted with extracts of three species of heterofermentative lactobacilli. Extracts of these aldolase-deficient bacteria provided an excellent control system and, as expected, produced no lines of precipitation with anti-SA (Fig. 13a).

Demonstration of partial antigenic homology with pediococcal aldolases. Cell-free extracts of the various strains of pediococci were compared in the same fashion as the lactobacilli. Exhibiting either confluent or single-spurred precipitates, the immunological comparisons detected three groups of apparent identical specificity. The first group is comprised of four strains of *P. acidilactici*, the second group contains *Pediococcus* sp. 559, *Pediococcus* sp. 990, *Pediococcus cerevisiae*, and *Pediococcus pentosaceus*, and the third group is represented by the aldolase of *P. parvulus*.

Circumscription of the family Lactobacillaceae. The relative ease with which the aldolase-antialdolase system detected intergeneric relationships among the three morphologically distinct groups of lactic acid bacteria made it essential to exclude the possibility of general or nonspecific reactions with aldolases of gram-positive bacteria not belonging to the *Lactobacillaceae*. To this end, cell-free extracts of *Staphylococcus aureus*, *Corynebacterium xerosis*, *Arthrobacter globiformis*, *Sporolactobacilus inulinus*, and *Microbacterium thermosphactum* were tested against the antisera. Only the aldolase of *M. thermosphactum*, a facultative psychrophile associated with meat spoilage (49), produced a precipitate; the others produced no visible cross-reaction (Fig. 13b).

The inclusion of this gram-positive rod in the survey was not accidental. Recently, a biochemical characterization of the genus *Microbacterium* by Collins-Thompson et al. (19) revealed that *M. thermosphactum*, unlike the other

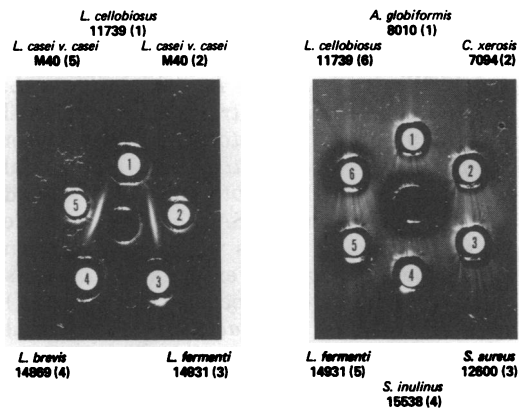


FIG. 13. *Results of cross-matches between aldolase-deficient lactobacilli or extracts of nonrelated gram-positive organisms. The sample well contains approximately 50 μg of crude extract protein; the center wells contain 40 μliters of anti-SA. The plates were incubated for 72 h.*

members of this loosely structured group, is a strictly homofermentative bacterium which lacks a tricarboxylic acid cycle. At a value of 36, the moles percent guanine plus cytosine ratio of its DNA is vastly different from the values obtained with the DNA of other species in the genus and is similar to that of most streptococci. Despite the similarities, the authors did not suggest that *M. thermosphactum* be assigned to the lactic acid bacteria. This reluctance stemmed from certain differences between *M. thermosphactum* and the lactobacilli; at variance were cell wall and lipid composition and the presence of catalase in the former. However, it is clear from the reaction between the *M. thermosphactum* aldolase and the anti-SA that the organism is related to the lactic acid bacteria.

No immunological evidence was found to

support the natural relationship proposed for *Sporolactobacillus* and *Lactobacillus* by Kitahara and Suzuki (36).

Evidence for antigenic divergence between aldolases of the lactic acid bacteria. The immunodiffusion experiments suggest that, within each genus, the aldolases of the constituent species have evolved in a relatively sequential fashion; the two exceptions found among the lactobacilli are described above. To determine whether any significant antigenic divergence had taken place at the intergeneric level, aldolases from each of the three genera were compared with one another. The creation of an immunologically based hierarchy made it possible to select aldolases from members of each genus whose reactivity with the antiserum was roughly equal. Several examples of such intergeneric comparisons are shown in Fig. 12a, b, and c. When extracts of *L. delbrueckii* or *L. lactis* were paired with extracts of streptococcal antigen group II (*Streptococcus cremoris* or *Streptococcus diacetilactis*) or streptococcal antigen group III (*Streptococcus bovis*) and made to react with anti-SA, double spurs were produced. Similar results were obtained when comparing *L. acidophilus* extracts with extracts of *S. salivarius* (streptococcal antigen group IV) or *Streptococcus dysgalactiae* (streptococcal antigen group V). The precipitation patterns obtained with enzyme extracts of *L. delbrueckii* and *S. salivarius* or *S. dysgalactiae* show single spurs directed towards the wells containing the enzyme with fewer antigenic determinants (Fig. 12a), whereas doubled-spurred bands were formed between extracts of *Pediococcus* sp. 990 and *L. lactis* (Fig. 12b), *Pediococcus* sp. 559 and *L. delbrueckii* (Fig. 12b, c), *L. xylosus* and *P. acidilactici*, and *M. thermosphactum* and *Pediococcus* sp. 559. Figures 14 and 15 summarize these double diffusion experiments, and from these data it is possible to discern at least four distinct lines of aldolase divergence. The data also show that many of the streptococcal aldolases are not as closely related to the *S. faecalis* enzyme as are aldolases found among several species of pediococci and lactobacilli.

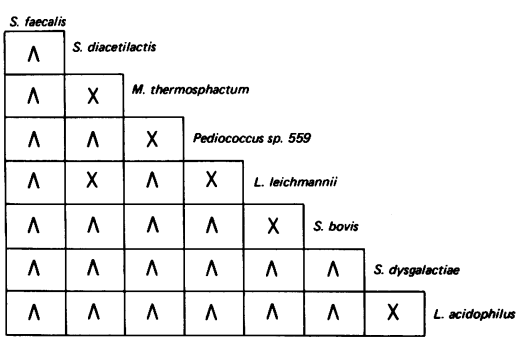


FIG. 14. *Summary of intergeneric cross-matches between the various aldolases of lactic acid bacteria.*

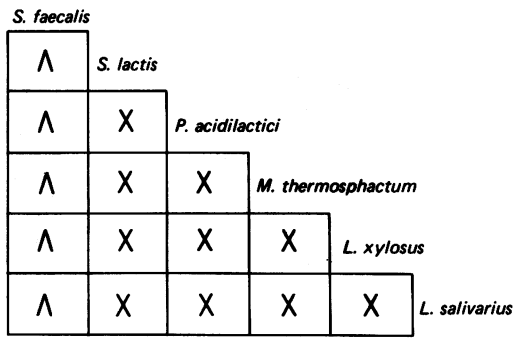


FIG. 15. *Summary of intergeneric cross-matches between aldolases from four morphologically distinct forms of lactic acid bacteria.*

Effect of anti-SA on aldolase activity. The addition of relatively small amounts (3 to 5 μliters) of anti-*S. faecalis* aldolase serum to reaction mixtures containing 0.2 to 0.4 units of aldolase present as either the pure enzyme or cell-free extracts produces a 50% inhibition of catalytic activity; the degree of inhibition is proportional to the antiserum concentration over the range of 1 to 10 μliters. Since the aldolase activity in extracts of *Streptococcus faecium*, *S. bovis*, *S. pneumoniae*, *Pediococcus* sp. 559, *L. casei*, *L. delbrueckii*, and *L. plantarum* is also sensitive to anti-SA, the inhibition by antiserum appears to be a general effect. Two types of controls serve to demonstrate the specificity of this system. First, substitution of equivalent amounts of unimmunized rabbit serum for anti-SA in the reaction mixtures did not result in an inhibition of activity. Second, the inhibition is specific for aldolases of lactic acid bacteria. The enzyme activity in extracts of *S. inulinus* is not inhibited by 40 μliters of anti-SA.

Although it is possible to establish an order of antigenic relatedness based on the extent of antiserum-induced inhibition of enzymatic activity (48), no attempt was made to quantitate the immunological differences between aldolases of the lactic acid bacteria by this method for the following reason. The two procedures employed in these experiments, immunodiffusion and microcomplement fixation, measure the total antibody-antigen interaction, whereas immune inhibition only measures the effect of such interactions on the catalytic site of the enzyme. This effect could conceivably be produced by antibody binding to only a few key antigenic sites on the enzyme molecule which

mask or modify the active site. Moreover, an amino acid substitution in one or more of the antigenic sites, which usually alters the configuration of the active site, could obliterate the inhibitory effect of the antiserum. In either of these circumstances, the total complement of antigenic determinants is probably not being measured.

Quantification of the Immunological Relatedness Among Aldolases of the Lactobacillaceae by Microcomplement Fixation

Determination of indices of dissimilarity by microcomplement fixation. The data from mCf assays were used to calculate the ID for the aldolases of the various lactic acid bacteria. These results both quantitate and corroborate those obtained by immunodiffusion experiments (Table 6). Based on their respective ID values, the aldolases were organized into clusters which generally coincided with the immunodiffusion groupings.

The streptococcal aldolases were segregated into five major clusters which, with only three exceptions, are comprised of the same enzymes found in the corresponding identity groups of the immunodiffusion experiments (compare Table 5 with Table 6, column 1). A clear separation of the individual clusters was achieved without further mathematical treatment of the data. In every instance the serological distance between the nearest neighbors within a specific cluster is always less than one-half the distance between the two neighboring clusters. The results also show that the constituent enzymes of each of the original five identity groups are not, in fact, immunologically homologous to one another; each cluster is composed of a spectrum of ID values rather than a single value. Even the aldolases of the first cluster, which corresponds to identical specificity group I, have undergone a measurable degree of antigenic divergence. The extent of antigenic divergence is more striking in the other clusters or groups of apparent specificity.

Only the aldolases of *Streptococcus mitis* 15909, *S. mitis* 15912, and *Streptococcus equisimilis* could not be accommodated within one of the five major clusters. The *S. equisimilis* aldolase is obviously less antigenically related to the reference protein than are the aldolases of apparent specificity group V. For the present, this enzyme has been used as a nucleus for a sixth cluster. The failure to detect spur formation when comparing the *S. equisimilis* aldolase with the group V aldolases can probably be attributed to the relative insensitivity of the immunodiffusion method. The *S. mitis* aldolases pose a special problem. Although *S. mitis* strains 15909 and 15912 can be assigned to a subgroup intermediate between apparent identity groups II and III (or clusters 2 and 3), the aldolase of a third strain, 15910, is clearly antigenically distinct from the two former enzymes. These data can be interpreted to mean that the species as presently constituted is an amalgam of heterogeneous organisms which do not belong under a single species heading. The situation is probably analogous to that described by Gasser et al. (27), in which the species *Lactobacillus jensenii* and *L. leichmannii* had been confused with one another because they were physiological look alikes.

An attempt was made to relate the antigenic groupings of the aldolases to the type of Lancefield antigen associated with the various species of *Streptococcus*. A rough correlation between the two antigenic systems can be seen from the data presented in Table 6, column 1.

A comparison of Fig. 11 with Table 6, column 2, reveals that the antigenic hierarchy of the *Lactobacillus* aldolases based on mCf assays is concordant with that of the immunodiffusion experiments. The results of the mCf studies establish unequivocally that the aldolases of the group D streptococci and *L. casei* share the greatest degree of immunological homology. In addition to the three *L. casei* subspecies, this cluster also contains the aldolases of *Lactobacterium zeae*, *Lactobacillus coryniformis* subsp. *coryniformis*, and *L. coryniformis* subsp. *torquens*. Since the immunodiffusion crossmatches of this group always produced lines of identity and the ID values cluster between 3.4 and 6.5 with an internal variation of 2.8 ID units, this group of aldolases appears to have a relatively high degree of antigenic homology. *L. zeae* has been characterized recently by Gherna and Mills (private communication) and has a phenotype identical with that of *L. casei* subsp. *rhamnosus*. The two subspecies of *L. coryniformis* differ from the conventional *L. casei* phenotype according to Abo-Elnaga and Kandler (1). Unlike *L. casei*, *L. coryniformis* subsp. *torquens* and *coryniformis* produce D and D,L-lactic acid, respectively, rather than L-lactic acid. At this time, the relative importance of these phenotypic differences cannot be assessed. The aldolases of *L. xylosus* and *L. salivarius* are shown as distinct entities in Table 6, column 2, in spite of their physical similarities; the precipitation pattern of nonidentity (double-spurred precipitate) between these two enzymes dictates such a separation. The order and aggregation of the remaining *Lactobacillus* aldolases are in accord with the hierarchical

TABLE 6. *Grouping of lactic acid bacteria aldolases according to their indices of dissimilarity*

Genus *Streptococcus*				Genus *Lactobacillus*			Genus *Pediococcus*		
Species			ID	Species		ID	Species		ID
S. faecalis	MR (homologous)	D	1.0	*L. coryniformis* subsp. *torquens*	M30	3.5 ± 0.1	*P. acidilactici*	25740	9.2 ± 0.6
S. faecalis	N83	D	1.1 ± 0.1	*L. coryniformis* subsp. *coryniformis*	M34	3.6 ± 0.2	*P. acidilactici*	25742	11.2 ± 1
S. faecium	K6A	D	1.0 ± 0.1	*L. casei* subsp. *pseudoplantarum*	M40	4.7 ± 0.1			
S. faecium	N55	D	2.1 ± 0.1	*L. zeae*	15820	4.7 ± 0.4	*Pediococcus* sp.	559	19.5 ± 0.6
S. durans	19432	D	2.7 ± 0.2	*L. casei* subsp. *rhamnosus*	F.3.4	4.7 ± 0.4	*P. cerevisiae*	8042	19.8 ± 1.5
				L. casei subsp. *casei*	64H	4.8 ± 0.2	*P. pentosacaceus*	25744	23 ± 3
S. lactis	19435	N	8.3 ± 0.5	*L. casei* subsp. *rhamnosus*	F.3.3.	5.0 ± 0.3	*Pediococcus* sp.	990	20 ± 2
S. cremoris	19257	N	9.0 ± 0.3	*L. casei* subsp. *rhamnosus*	OC91	5.4 ± 0.6			
S. thermophilus	19258		10.4 ± 0.7	*L. casei* subsp. *alactosus*	OC45	5.8 ± 0.2	*P. parvulus*	19371	39 ± 1.8
S. diacetilactis	11007	N	13.5 ± 1	*L. casei* subsp. *rhamnosus*	CL11	6.3 ± 0.7			
S. mitis	15909		18 ± 1	*L. xylosus*	15577	20 ± 2	Others		
S. mitis	15912		25 ± 4						
				L. salivarius	11741	30 ± 4			
S. bovis	9809	D[a]	34 ± 1				*Microbacterium thermosphactum*	11509	11.7 ± 0.4
S. bovis	15351	D[a]	39 ± 2	*L. bulgaricus*	11842	36 ± 2			
S. sanguis	10556	H	39 ± 2	*L. lactis*	12315	39 ± 8			
S. equinus	9812	D[a]	39 ± 3	*L. delbrueckii*	9649	41 ± 5			
S. pneumoniae	6308		39 ± 6	*L. leichmannii*	4797	43 ± 3			
S. mutans	K1R		42 ± 0.5						
				L. curvatus	M1	116 ± 7			
S. asalignus	8059	B	56 ± 7						
Streptococcus sp.	8144	*H*	61 ± 7	*L. mali*	27053	133 ± 3			
S. agalactiae	13813	B	66 ± 9						
S. salivarius	112	K	66 ± 3	*L. plantarum*	14917	138 ± 7			
S. salivarius	9222	K	66 ± 9						
S. salivarius	13419	K	70 ± 5	*L. acidophilus* (Ib)	19992	176 ± 8			
S. pyogenes	14298	A	80 ± 7	*L. acidophilus* (III)	4356	203 ± 20			
S. mitis	15910		80 ± 8						
S. equi	6580	C	81 ± 9	*L. jensenii*	25258	225 ± 24			
Streptococcus sp.	9932	L	82 ± 8						
S. dysgalactiae	9926	C	88 ± 13	*L. jugurti*	521	268 ± 21			
				L. helveticus	15009	290 ± 11			
S. equisimilis	9542	C	105 ± 7						

[a] Reacts with group D antisera, but possesses antigens not found associated with group D *Streptococci*.

scheme proposed in an earlier section and requires no further explanation here. Two organisms, *Lactobacillus curvatus* and *Lactobacillus mali*, were added to the list of bacteria surveyed by mCf assay. Both aldolases appear to be intermediate between the *L. leichmannii* and *L. acidophilus* groups; these positions were subsequently confirmed by immunodiffusion tests.

As was the case with the *Lactobacillus* aldolases, the arrangement of the pediococcal aldolases in accordance with either their ID values or reactions of partial identity was identical. The three clusters shown in Table 6, column 3, are coincident with the three groups of apparent identical specificity.

With ID values of approximately 10, the degree of homology between the group D streptococcal aldolases and the aldolases of the group N streptococci, *P. acidilactici* and *M. thermosphactum*, appears to be equal. Since the aldolases of the three organisms belong to different molecular weight classes and produce lines of nonidentity when compared with one another on immunodiffusion plates, it seems safe to conclude that they represent three distinct lines of aldolase evolution.

DISCUSSION

Molecular Variation During the Course of Aldolase Evolution

It has become general practice to estimate the extent of structural similarity of isofunctional proteins by comparing one or more of their various physical characteristics; the two properties most frequently used are the electrophoretic mobility coefficient and molecular weight. Closely related or homologous proteins are usually invariant, or nearly so, in either parameter; conversely, variability is associated with a decrease in structural homology. Do the FDP aldolases of lactic acid bacteria constitute an exception to this well-established rule? The striking diversity in size and protein charge of the aldolases found among the three genera of lactic acid bacteria would suggest that very little structural homology exists between the physically distinct forms of the enzyme. This is obviously not the case, however; in addition to establishing the existence of structural homology between the various groups of aldolases, the results of the immunological studies suggest that the degree of structural homology is virtually unrelated to the above-mentioned two major physical properties of these proteins. A comparison of the *S. faecalis* and *L. casei* aldolases best typifies this anomaly. The *L. casei* aldolase is twice as large and one-half as mobile in PAG columns as the reference *S. faecalis* enzyme but, despite these differences, the former shares a degree of immunological homology with the latter which is surpassed only by aldolases of other Lancefield group D streptococci. Fortunately, a relatively simple explanation of this apparent enigma can be extracted from the available data. Dividing the average molecular weight of each of the five aldolase size classes (see Table 2) by 28,000, the molecular weight of the *S. faecalis* aldolase subunit, the integers 2, 3, 4, 5, and 6 are obtained. If the assumption is made that the various representatives of five molecular weight classes of aldolase simply represent the association of two or more identical subunits, the results of the physical and immunological studies are no longer incongruent. The variations in molecular weight merely reflect the different modes of assembly of the structurally conserved subunit, and since the polyacrylamide gels also act as molecular sieves (13) the electrophoretic mobility rates are influenced, at least in part, by the size of the enzyme. Although this point of view is generally consistent with the data, the possibility that the net protein charge has been altered by amino acid substitutions in nonconserved areas of the peptide chain cannot be excluded. Implicit in this hypothesis is the assumption that subtle mutations in the primary amino acid sequence of the monomer are responsible for the gross alterations in the quaternary structure of the enzyme. There is already some evidence from preliminary studies with the aldolases of *L. casei* and *P. cerevisiae* (unpublished data) to indicate that these enzymes are, in fact, composed of subunits arranged in a tetrameric and hexameric configuration, respectively.

Although variations of this magnitude in the quaternary structure of partially homologous proteins are quite rare, such occurrences are not completely without precedent. Sadoff and coworkers (66) found that the FDP aldolase from spores of *Bacillus cereus* is roughly one-half as large as the major molecular weight species of aldolase present in the vegetative cell, yet antisera prepared against the purified spore enzyme produced confluent lines of precipitation when the former was compared with the larger aldolase of the vegetative cell on immunodiffusion plates. This result indicated that the two forms of the enzyme are immunologically homologous. A more detailed description of the molecular evolution of the lactic acid bacteria FDP aldolases will be presented in a separate report.

Preparation of a Phylogenetic Map for the Homofermentative Lactic Acid Bacteria Based on Structurally Related Proteins

Basis for relating immunological homology to structural homology. The rationale for using immunological techniques to measure amino acid sequence homology of isofunctional proteins has been amply discussed by a number of workers, including Wilson and Kaplan (84), Prager and Wilson (60), Stanier et al. (72), Gasser and Gasser (26), and London et al. (43, 44), and a detailed reiteration of these points is not necessary here. However, several issues related to the precision of the methodology, its limitations, and the restrictions these factors place upon the interpretation of the results deserve elaboration at this time. Studies with proteins of known amino acid sequence have established that quantitative immunological techniques can readily detect substitutions of one (14, 61), two (6, 7), or three (5) amino acids in the primary structure of partially homologous proteins. The superb sensitivity of the microcomplement fixation assay prompted Prager and Wilson (60) to suggest that the immunological distance (log of the index of dissimilarity $\times$ 100) between two related proteins can be correlated with a specific number of amino acid substitutions in the primary structure of the proteins. By using avian egg white lysozymes as a model system, these workers established that five immunological distance units are equivalent to an alteration of one amino acid. The authors went on to suggest that this relationship may be generally applicable to all species of proteins if their stringent experimental procedures are followed.

The general attitude towards the suitability of using immunological procedures to estimate protein homology is somewhat less enthusiastic than that cited above and is probably best expressed by some concluding remarks in a report by Margoliash et al. (47): "With eucaryotic cytochrome C it was found that the correlation of primary structure and immunological cross-reaction is, in general, good but not perfect. This probably reflects the fact that not all structural changes in cytochrome C, only those that are effectively within the antigenic determinants, effect the immunological reaction. . . . Despite these limitations, immunological cross-reactions among sets of ancestrally related proteins from different species may provide useful taxonomic indications."

Until the recent report by Prager and Wilson (60), little was known of the extent to which the structures of related proteins could be altered and still retain immunological cross-reactivity. Based on their own work and a survey of the literature, these authors found that a primary sequence difference of 25 to 40% resulted in a complete loss of immunological activity in all species of proteins examined thus far. A further restraint is placed on the usefulness of the immunological approach by the knowledge that these techniques only measure the similarities in the surface or external portions of the proteins, and it is precisely these areas which tend to evolve most rapidly (46, 60). Hence, the immunological procedures appear to be limited to relating sets of proteins with relatively high sequence homologies (60% or greater).

The number of antigenic determinants found on a protein can also influence the reliability of immunological homology data. For example, if a group of structurally related proteins possess only a few antigenic determinants per molecule, any alterations in those sites could greatly exaggerate the differences between the actual sequence homology and that determined by immunological tests. In these instances, the degree of sequence homology might be relatively great, although the immunological techniques would indicate that little or no homology exists between the various ancestrally related proteins.

Despite its limitations and possible pitfalls, it will become apparent from the ensuing discussion that immunological procedures provide a rapid, convenient, and reliable means of comparing the structures of isofunctional proteins.

Phylogenetic map of the lactic acid bacteria. The phylogenetic map of the homofermentative lactic acid bacteria (Fig. 16) was assembled in four stages by using both physical and immunological properties of the FDP aldolases. (i) The immunodiffusion patterns of nonidentity delineate the branches of the four major diverging lines of aldolase evolution and provide the backbone of the scheme (solid lines, Fig. 16). (ii) Within each of the lines, the positions of the individual species are first approximated by the immunodiffusion reactions of partial identity; (iii) a more precise placement is made possible by quantifying the distances along each line by using the microcomplement fixation data (ID values). (iv) Finally, minor diverging branches (dotted lines) corresponding to the different aldolase molecular weight classes are included. The significance of these subgroups is as yet unknown. Since the size of the enzyme appears to be a function of subunit arrangement, the differences in molecular weight may represent little more than the substitution of several amino acids in the primary structure of the enzyme. However, these apparently trivial differences may produce functional changes in a

particular size class of aldolase which confer a selective advantage upon the organisms possessing them.

The results of using these criteria for charting evolutionary relationships are readily apparent in Fig. 16. For example, the reactions of nonidentity (double-spurred precipitates) between the aldolases of *L. xylosus* and *L. salivarius* necessitate their placement on two distinct and diverging lines of evolution despite the fact that these aldolases belong to the same molecular weight group. Differences in the molecular weights of their aldolases have resulted in the placement of *S. salivarius*, *P. parvulus*, *L. plantarum*, and *L. curvatus* in minor evolutionary outcroppings.

Although a large number of organisms have been examined thus far, this map cannot be considered complete. With the exception of *Butyribacterium rettgeri* (unpublished data), the obligately anaerobic gram-positive nonsporeforming rods have not yet been surveyed. At this juncture it should be pointed out that the map will probably have to be enlarged since the aldolase of *B. rettgeri* reacts strongly with the antistreptococcal aldolase serum. The relationships between this large group of gram-positive rods and the homofermentative lactic acid bacteria shall be treated in a separate report.

Does the Phylogenetic Map Accurately Reflect Interrelationships Among the Lactobacillaceae?

Phylogenetic maps of the lactic acid bacteria as elaborate as this one have not yet been assembled; however, Gasser and Gasser (27) have used D- and L-lactic dehydrogenases (LDH) to chart the natural affinities which exist among a large number of species of homo- and heterofermentative lactobacilli, and a part of their map overlaps with the aldolase map. A reproduction of one of their maps is shown in Fig. 17, and this is to be compared with the main *Lactobacillus* branch of the aldolase map (Fig. 16). If the two branches of homofermentative lactobacilli are rotated 90° towards each other to form a single linear sequence, the order of the constituent species in the sequence *is essentially identical* with that found in the FDP aldolase map. Moreover, both immunological systems recognize the same groups of identical

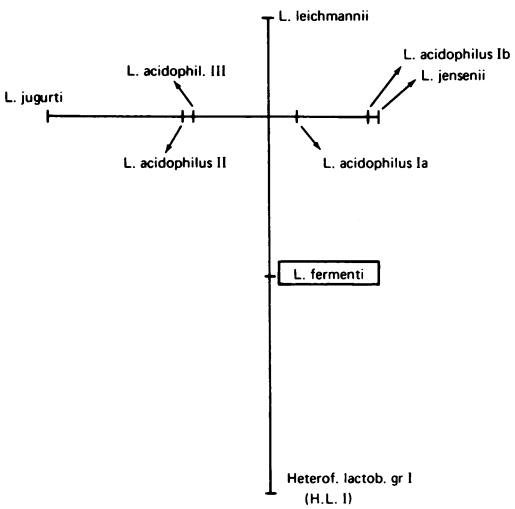


FIG. 17. *Antigen relationships of Lactobacillus LDH based on microcomplement fixation studies (after Gasser and Gasser, reference 26).*

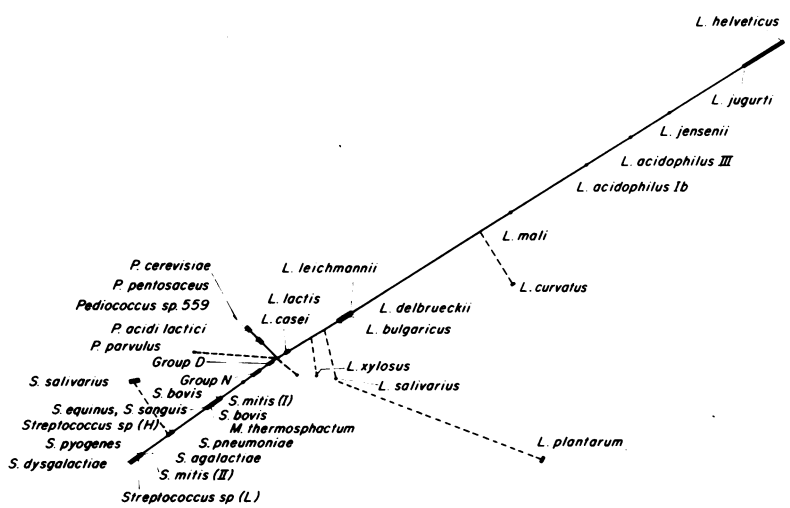


FIG. 16. *Proposed phylogenetic map of the homofermentative lactic acid bacteria.*

specificity, namely, the *L. leichmannii* group consisting of *L. leichmannii*, *L. bulgaricus*, *L. lactis*, and *L. delbrueckii*, and the *L. jugurti-L. helveticus* group. The failure to detect the two diverging lines of lactobacilli with the anti-streptococcal aldolase serum can probably be attributed to the relatively great immunological distance between the aldolases of the *L. acidophilus* groups and the reference protein. The antigenic determinants which these enzymes now share are probably few in number and highly conserved. The discrepancy in the two sets of data emphasizes the importance of the internal reference protein. By using a group-specific aldolase or an aldolase of a closely related *Lactobacillus*, the divergence at the *L. acidophilus* groups would probably be detectable. On the basis of weak cross-reactions with anti-*L. acidophilus* L-LDH serum, the authors concluded that the LDH of *L. casei* and *L. salivarius* are related, but immunologically distant from the *L. leichmannii* group. This observation is also in accord with the aldolase map. The inability of the anti-L-LDH serum to cross-react with the LDH of the group D streptococci can be explained simplistically by suggesting that the antiserum titer was comparatively lower than that of the anti-aldolase serum. However, the possibility cannot be excluded that the FDP aldolase is a more structurally conserved protein than the L-LDH and therefore evolves at a lower rate.

Although it is apparent that portions of both schemes require much refinement, it is equally important to acknowledge the fact that those areas of the maps which overlap exhibit a high degree of coordination. The excellent agreement in the ordering of nine species of *Lactobacillus* permits both maps to be interlocked and the homofermentative streptococci and lactobacilli can readily be related to a number of aldolase-less heterofermentative species of *Lactobacillus* and *Leuconostoc*. A precise integration of the two maps is not possible at this time, since the immunological distances of the aldolases are probably not equivalent to those of the lactate dehydrogenases. Despite this limitation, the aldolase map can be united with the expanded LDH map of Gasser and Gasser (26) (Fig. 18) to reveal the evolutionary sequence of the constituent species. From the points of overlap, the homofermentative members of this family spread out in one general direction, whereas the heterofermenters lie in the opposite direction; the diverging groups form two large discreet clusters. Studies with other conserved proteins will be needed to extend and confirm both phylogenetic maps.

Implications of the Phylogenetic Map

Evolutionary interrelationships. For the present, no significance should be attached to the direction of the respective evolutionary branches. The spacing and distance between each of the lines merely represent the most convenient way of displaying the data. Similarly, the data are presented in a two-dimensional plane, and the arrangements within groups of *apparent identical specificity are probably three dimensional.* Without internal markers, it is not possible to arrange these antigenically related groups with a high degree of confidence. For this reason, the positions within groups of apparent identical specificity are drawn in a linear fashion. The map does show that the four genera are related through some common ancestor, and it allows the relative evolutionary distances between groups and individual species to be easily determined. However, the most provocative aspects of the map are not readily apparent, and these are discussed below.

Morphology versus protein homology. It can be seen from Fig. 16 and Table 6 that *S. faecalis* and *L. casei* form a bridge between the rods and the cocci; but, in addition to this fact, it is also apparent that the latter is the species most closely related to the group D streptococci. Because one species is rod shaped and the other spherical, a dilemma involving a basic precept in microbial taxonomy immediately arises. How important a taxonomic character is the shape of microorganisms? Clearly, in schemes not reflecting natural relationships a property of this sort can assume any degree of importance, and it may provide a convenient method of distinguishing two groups of organisms whose physiological characteristics are very similar. If, however, the protein homology studies of the malic enzyme (43, 44) and aldolase accurately reflect the natural affinities between the species *L. casei* and *S. faecalis*, then one must conclude that shape is a relatively trivial character which should not be used to divide closely related groups.

The morphological differences which exist between the tetrad, *P. acidilactici*, and the *Streptococcus*, *S. faecalis*, also appear to be of minor importance because the immunological distance between *P. acidilactici* and *S. faecalis* is the same as that between the latter and *S. lactis*, its nearest streptococcal neighbor (see Table 6). The same relationship can be shown for the three species, *S. faecalis*, *M. thermosphactum*, and *S. lactis*. Unlike *Arthrobacter* species (39), the lactic acid bacteria breed true,

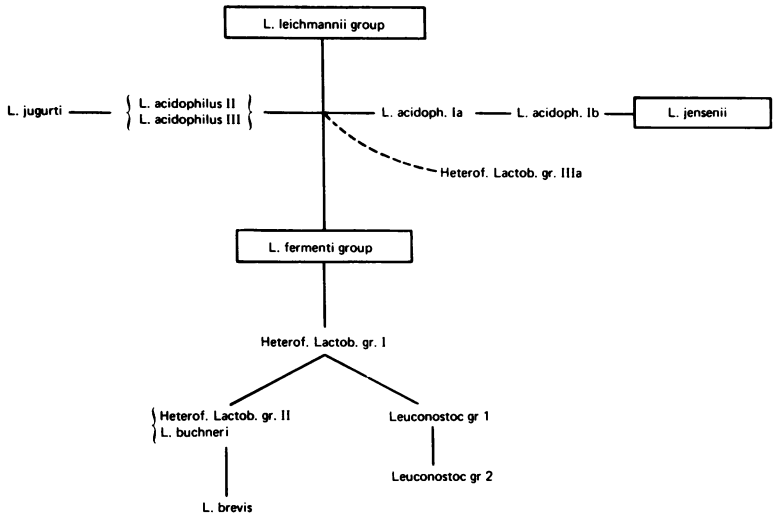


FIG. 18. *Interrelationships between heterofermentative Lactobacillus and Leuconostoc species based on immunological similarities of their LDH (after Gasser and Gasser, reference 26).*

rods producing rods and spheres producing spheres. However, it now appears that the establishment of a stable spherical or rod-shaped genotype among this group of organisms may involve relative minor genetic mutations. The argument has been framed succinctly by van Niel (77), who wrote: "To believe that mere resemblance in morphology represents a sound basis for revealing phylogenetic trends—as is implicit in the arguments of priority of morphological over physiological characteristics—rests on a misconception of the fundmental value of such characters when successfully employed for the classification of higher plants and animals, where they are intricately linked with the entire developmental history of the organisms."

Comparative evolutionary age of the respective genera. If it is assumed that (i) isofunctional sets of prokaryotic proteins are similar to isofunctional sets of eukaryotic proteins and evolve at relatively constant rates, and (ii) that the rate of amino acid substitution within the antigenic determinants accurately reflects the rate of amino acid replacement of the entire protein, it is possible to use the immunological distance as a rough measure of relative evolutionary time. The microbial line exhibiting the greatest immunological divergence as manifested in the span of ID values should be considered the most ancient group in the family. Among the lactic acid bacteria, the genus *Lactobacillus* is the most immunologically diverse group found and is, therefore, the oldest group in this family. The streptococci appear to be a more recent group, whereas the pediococci exhibit the least divergence of the three morphologically distinct groups. This line of reasoning leads to a rather obvious conclusion, namely, that the rod-shaped forms are the progenitors of the spherical forms. Benecke (10) postulated that the coccoidal forms are the simplest morphological entities in nature, and that these gave rise to the more complex tetrad and rod-shaped bacteria; the evidence presented here argues against this point of view. However, the conclusions must be qualified. Although the survey of species belonging to the *Lactobacillaceae* was comprehensive, it is possible that there are streptococci in nature which possess aldolases with a greater degree of evolutionary divergence than those studied here, and these organisms would expand the evolutionary time scale of this genus.

Protein Relatedness Studies Among Other Bacteria

Enterobacteriaceae. Five species of isofunctional enzymes have been used to demonstrate the natural relationships which exist among the various genera of enteric bacteria. Cordes and Levine as cited by Wilson and Kaplan (84) prepared antisera against the purified inducible alkaline phosphatase of *Escherichia coli* and established the following order of relatedness of the four genera studied: *Escherichia* > *Aerobacter* > *Serratia* > *Proteus*. A comparative immunological study of the tryptophan synthetase α subunit (53) of a number of enteric bacteria with antiserum prepared against the *E. coli* α subunit revealed that the five genera

under investigation were related in the order: *Escherichia* > *Shigella* > *Salmonella* > *Aerobacter* > *Serratia*. Recently, Cocks and Wilson (15) confirmed and extended the work of Cordes and Levine by comparing the alkaline phosphatases of a larger number of *Enterobacteriaceae* with antisera prepared against the pure alkaline phosphatases of *E. coli* and *Klebsiella aerogenes*. A much enlarged phylogenetic map showed the following intergeneric relationships: *Escherichia* > *Shigella* > *Salmonella* > *Citrobacter* > *Klebsiella* > *Serratia* > *Proteus* > *Erwinea*. Where the three sets of data overlap, the ordering of the genera is identical. The patterns of immunological cross-reactivity observed when comparing the enteric β-galactosidases (18, 64), L-asparaginases (59), and the T and G factors involved in protein synthesis (28) are in general accord with the results cited above. Taken together, the five studies circumscribe and define a phylogenetic group composed of eight bacterial genera. The data are also consistent with DNA homology studies (11).

The alkaline phosphatases from species within the genus *Klebsiella* have been compared immunologically by Steffen et al. (73). These studies indicate that *K. aerogenes* and *Aerobacter* (*Enterobacter*) *aerogenes* are one and the same organism. However, the genus *Enterobacter* appears to be relatively heterogeneous, containing species such as *Enterobacter liquefaciens*, which are only distantly related to the reference strain *E. aerogenes*.

Pseudomonadaceae. Stanier and co-workers prepared antisera against purified muconolactonizing enzyme (MLE) and muconolactone isomerase (MI) from *Pseudomonas putida* biotype A in an attempt to clarify the taxonomic structure of this genus. Although the isologous enzymes found in *Pseudomonas multivorans* and *Pseudomonas acidovorans* did not cross-react with either of the antisera, the MLE of *Pseudomonas aeruginosa*, *Pseudomonas stutzeri*, and *Pseudomonas fluorescens* biotypes A, B, C, D, and E reacted definitively with the anti-*P. putida* MLE. In addition, extracts of a number of taxonomically indeterminate strains of *Pseudomonas* also reacted with the anti-MLE serum. Only three of the species possessed MI which reacted with the anti-*P. putida* MI serum. On the basis of this study, the authors excluded the organisms designated *P. putida* biotype B from the species.

Micrococcaceae. A comparative study by Whiteside et al. (82) of a membrane-bound adenosine triphosphatase (ATPase) found among Gram-positive aerobic cocci revealed that the micrococci and certain *Sarcina* are related. As in the case of the gram-positive homolactic cocci, the morphological differences between *Micrococcus* and *Sarcina* do not constitute a natural division or boundary of this group. The ATPase of *Sarcina flava* and *Sarcina lutea* reacted strongly with the *Micrococcus lysodeikticus* anti-ATPase serum, as did many of the *Micrococcus* species. The micrococci were arranged in the following order of relatedness: *M. lysodeikticus* > *Micrococcus tetragenus* > *Micrococcus roseus* > *Micrococcus conglomeratus* > *Micrococcus varians*. The failure of the ATPase from *Micrococcus rhodochrous*, *Sarcina ventriculi*, *Sarcina maxima*, and *Sporosarcina ureae* to react with the anti-ATPase serum came as no surprise to the authors. Of the organisms studied, only *Micrococcus caseolyticus* remained taxonomically indeterminate; *M. rhodochrous* phenetically resembles the actinomycetes and had already been reassigned to that family (29). Similarly, the anaerobic sarcinae had been clearly differentiated from their aerobic counterparts by Canale-Parola (12) and have since been relegated to the family *Peptococcaceae* (63). Finally, the authors pointed out that the *S. ureae* properly belongs among the gram-positive sporeforming bacteria.

Suprafamilial relationships. Thus far, the discussion has centered on the establishment of natural relationships among organisms within specific taxonomic families (as they are defined in *Bergey's Manual of Determinative Bacteriology*, 7th ed.). However, several recent studies indicate that it may be possible to demonstrate interfamilial relationships. McFadden and Denend (48) prepared antiserum against pure *Hydrogenomonas eutropha* ribulose diphosphate (RUDP) carboxylase and showed that it would inhibit RUDP carboxylase activity in extracts of *Rhodospirillum rubrum*, *Chlorella pyrenoidosa*, *Chromatium* D, and *Thiobacillus denitrificans* to varying degrees. Unfortunately, immune precipitates were not produced when the various extracts were made to react with anti-RUDP carboxylase serum in immunodiffusion experiments. The haptene-like behavior of the antigen suggests that the RUDP carboxylases of the respective photo- and chemolithotrophs share very few antigenic determinants with the homologous protein.

Tronick et al. (76) reported that the glutamine synthetase of nine diverse species of gram-negative organisms shared antigenic determinants with the isofunctional enzyme of *E. coli*. The anti-*E. coli* glutamine synthetase serum reacted with the partially homologous enzymes

in the order *Enterobacteriaceae* > *Pseudomonadaceae* > *Azotobacteriaceae* (private communication, S. Tronick and J. Ciardi). Glutamine synthetases of several gram-positive organisms did not react with the antiglutamine synthetase serum, indicating that the divergence which ultimately produced gram-positive and gram-negative organisms is beyond the limits of detection of this immunological system and is, therefore, probably relatively ancient. These two preliminary reports suggest that among prokaryotes there exist enzymes with highly conserved primary structures. Studies of such enzymes may permit bacteriologists to eventually prepare phylogenetic maps which encompass many of the known bacterial families.

Rates of Evolution of Prokaryotic Proteins

Noting that the indices of dissimilarity for several sets of isofunctional enzymes found in enteric bacteria were very similar, Cocks and Wilson (15) theorized that intracellular metabolic enzymes might evolve approximately in unison. Shortly thereafter, Rocha et al. (62) demonstrated that this was by no means a general rule. Within the same protein, tryptophan synthetase, the α and β_2 subunits from members of five genera of enterobacteria were shown to evolve at markedly different rates by immunological procedures.

Stanier and co-workers (72) found a similar situation among enzymes from the pseudomonads. The inducible muconate-lactonizing enzyme appears to have sustained fewer antigenic alterations than its companion enzyme, muconolactone isomerase. Although the titers of the antisera prepared against each of the *P. putida* enzymes were essentially identical, the antimuconolactone isomerase serum reacted with far fewer extracts of *Pseudomonas* species than did the antimuconate-lactonizing enzyme serum.

It also appears that catabolic enzymes of the lactic acid bacteria may not evolve in unison. The species *L. casei* can be easily differentiated into its two constituent subspecies, *L. casei* var. *casei* and *L. casei* var. *rhamnosus*, by a series of physiological tests (44). Similarly, antisera prepared against the inducible malic enzymes of either *S. faecalis* or *L. casei* var. *casei* recognize two antigenically distinct forms of enzyme among members of the species *L. casei* (Table 7). One of the antigenic forms of the malic enzyme is found only in *L. casei* var. *casei*, and the other form is found only in strains of *L. casei* var. *rhamnosus*. DNA homology data are in complete agreement with this division of the species (52, and J. Johnson, private communication). In sharp contrast, the FDP aldolase ID values are relatively uniform and cannot be used to differentiate the two subspecies (Table 6). From the lower indices of dissimilarity and absence of a clear divergence between the *L. casei* subspecies, it is clear that the constitutive FDP aldolases are evolving at a significantly slower rate than either the inducible malic enzyme or gross nucleotide sequence of the genome.

TABLE 7. *Comparison of the indices of dissimilarity of malic enzyme and FDP aldolase*

Strain tested	ID for malic enzyme			ID for Aldolase
	Anti-*S. faecalis* MR malic enzyme	Anti-*L. casei* 64H malic enzyme	Anti-*L. casei* M40 malic enzyme	Anti-*S. faecalis* FDP aldolase
Streptococcus faecalis MR	1.0	20	19	1.0
Lactobacillus casei var. *casei* 64H	52	1.0	1.0	4.8
L. casei var. *casei* M40	51	1.0	1.0	4.7
L. casei var. *alactosus* OC45	44	1.0	1.0	5.8
L. casei var. *rhamnosus* OC91	78	5.7	7.2	5.4
L. casei var. *rhamnosus* CL-11	72	11	9.4	6.3

CONCLUSIONS

The absence of paleontological records and intricate development histories can no longer deter the microbiologist from establishing natural relations between physiologically and morphologically distinct groups of prokaryotes. The bacterium's macromolecules, i.e., DNA, RNA, and proteins, have become the "fossil records" with which contemporary taxonomists are charting natural affinities in the microbial world. In contrast to similar studies with eukaryotic proteins, there is no way at present to ascertain whether a species of bacterium is of recent or ancient origin. To determine the age of a species of organism or protein, paleontological records are needed as reference points in time. For the present, microbial taxonomists must content themselves with the preparation of phylogenetic maps with only comparative time scales. Relative time scales can be based on immunological or amino acid sequence differences. However, within this limited framework,

the amount of work to be done is formidable.

Our present level of technology is such that DNA homology studies can provide the basis for a realistic and modern definition of a bacterial species (see Stanier, reference 71). Once a contemporary species concept has been formulated, relatedness studies similar to this one can be integrated in such a way as to define the higher taxonomic units. Thus, infraspecific and infrageneric relationships can be established by comparing the nucleotide base sequence of DNA molecules or rapidly evolving proteins. More conserved proteins will be useful in demonstrating relationships at the tribal or family level. Suprafamilial groupings may be achieved by comparing highly conserved proteins or determining RNA homology. A classification system based upon these criteria will also have the advantage of permitting microbiologists to detect the creation of new microbial species as well as following the evolution of well-established lines of bacteria.

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ADDENDUM

Subsequent to the completion of this manuscript, a paper by S. Tafler, P. Setlow, and L. Levine describing an immunological study of bacterial DNA polymerases was published (J. Bacteriol. **113**:18, 1973). Antisera prepared against homogeneous *E. coli* DNA polymerase I were used to determine the degree of immunological relatedness between similar enzymes found in other enteric bacteria. The following order of relatedness can be ascertained from the reported indices of dissimilarity: *Escherichia* = *Shigella* > *Salmonella* > *Klebsiella* > *Aerobacter* > *Serratia* = *Erwinia* > *Proteus*. This ordering differs from the others cited in this manuscript only in the placement of *Erwinia* and *Proteus*.

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